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Molecular farming of antimicrobial peptides: available platforms and strategies for improving protein biosynthesis using modified virus vectors

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Abstract: The constant demand for new antibiotic drugs has driven efforts by the scientific community to prospect for peptides with a broad spectrum of action. In this context, antimicrobial peptides (AMPs) have acquired great scientific importance in recent years due to their ability to possess antimicrobial and immunomodulatory activity. In the last *two decades, plants* have attracted the interest of the scientific community and industry as regards their potential as biofactories of heterologous proteins. One of the most promising approaches is the use of viral vectors to maximize the transient expression of drug*s in the leaves of the p*lant Nicotiana benthamiana. Recently, the MagnifectionTM expression system was launched. This sophisticated commercial platform allows the assembly of the viral particle in leaf cells and the systemic spread of heterol*ogous protein* biosynthesis in green tissues caused by Agrobacterium tumefaciens "gene delivery method". The system also presents increased gene expression levels mediated by potent viral expression machinery. These characteristics allow the mass recovery of heterologous proteins in the leaves of N. benthamiana in 8 to 10 days. This system was highly efficient for the synthesis of different classes of pharmacological proteins and contains enormous potential for the rapid and abundant biosynthesis of AMPs.

Key words: antimicrobial peptides, Magnifection, *Nicotiana benthamiana*, transient expression.

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INTRODUCTION

Historically, pathogenic microorganisms represent the greatest health risk for individuals whose immune system is compromised by milder diseases or by invasive therapies (Watkins and Bonomo 2016). The need to attenuate or even prevent the proliferation of these microorganisms in the hospital environment has been a constant concern of leading scientific research groups around the world (Uhlig et al. 2014). Many of the pathogens commonly found in hospitals are resistant to antimicrobial drugs commonly used in the treatment of common or high-risk conditions. The indiscriminate use of antibiotics and antifungals without a medical prescription has led, since the mid-1960s, to a consistent increase in the isolation of resistant strains in hospitals, especially in Latin America (Andersson et al. 2016).

The high rate with which resistant pathogens are selected throughout the treatment of patients is still the main problem regarding the use of conventional antimicrobials. In this scenario, many of the most common, cheap and once-effective pathogen control drugs become ineffective in systemic infection (Perry et al. 2016). Due to the exponential growth of cases of antibiotic-resistant microorganisms, the development of new control alternatives and molecules with broad action spectrum and low toxicity has acquired a strategic character for the pharmaceutical industry (Kosikowska and Lesner 2016, Nagel et al. 2016, Andersson et al. 2016).

In this context, antimicrobial peptides (AMPs) present themselves as a valuable alternative in the complementation or substitution of traditional therapeutic compounds. AMPS are natural antibiotics found in microorganisms, plants and animals, which function as important components of the innate immune system, the first line of defense of these organisms against exogenous pathogens. In the early stages of infection, molecular stimuli emitted by intrinsic signaling systems trigger the biosynthesis of AMPS in order to cope with the invading agent (Mojsoska and Jenssen 2015).

From the structural point of view, AMPs are characterized as small protein molecules with less than 100 amino acid residues, often rich in cysteine, glycine or histidine residues and which can be classified according to the presence of α-helices, β-sheets, extensions in the side chains of the nucleus amino acids or by the formation of disordered loops (Cunha et al. 2017). These small peptides can also be grouped according to their physicochemical properties in anionic, cationic (usually with positive charges between 2 to 9) and amphipathic AMPs, with their hydrophobic and hydrophilic portions placed side by side (Nguyen et al. 2011, Malanovic and Lohner 2016).

The first function of AMPs is to kill or inhibit the growth of microorganisms at the molecular level. In general, the classical mode of action of cationic AMPs is due to the structural disorganization and formation of pores in the membrane, with the consequent rupture of the lipid bilayer that delimits fungal cells and Gram positive and negative bacteria (Perry et al. 2016). For this reason, AMPs can act against different cell targets and in some cases, be considered "promiscuous" molecules, capable of performing more than one function, regardless of their structure. Recent reports have shown that some AMPs can destabilize translational machinery (ribosomes) and inhibit DNA synthesis and the cell cycle of certain fungi and bacteria (Nguyen et al. 2011, Malanovic and Lohner 2016). Some of these molecules can still act as antivirals (Malanovic and Lohner 2016), as insecticides and as hemolytic and antitumor agents (Nguyen et al. 2011, 2012).

The biochemical and physicochemical characteristics inherent to the structure of the AMPs are crucial for the types of biological functions of these molecules. The length and distribution of charged groups along the peptide chain, degree of hydrophobicity, N-terminal amidation, and the type

and number of secondary structures determine the efficiency of the bioactive peptide and its cytotoxic ability to different cell types and microorganisms (Nguyen et al. 2011).

Among the several AMPs already characterized, one class in particular has a special value for medicine: the innate defense peptides (host defense peptides) (HDPs). These small 12- to 50-amino acid peptides are generally non-toxic to mammalian cells and can be isolated from unicellular, invertebrate microorganisms such as mollusks, plants, amphibians, birds, fish and mammals (including humans). HDPs conjugate the direct antimicrobial action with the modulation of the innate immunity of the host organism through the induction of the inflammatory process. Thus, when synthesized in response to microbial action, HDPs activate cytokine synthesis, the occurrence of apoptosis and chemotaxis, the recruitment and proliferation of macrophages, neutrophils, eosinophils and T lymphocytes, as well as differentiation of dendritic cells (Silva et al. 2011).

The diverse functions that the HDPs can simultaneously present and their degree of selectivity reflect the enormous potential of use of these molecules for the protection of patients. This phenomenon of "promiscuity and functional dynamism" is characterized as the basic principle for the design of new drugs and for the preparation of biotechnological studies with AMPs candidates to act as new antibiotics (Pelegrini et al. 2007).

THE HETEROLOGOUS EXPRESSION OF AMPS AIMED AT IMPROVING THE FIGHT AGAINST FUNGAL AND BACTERIAL INFECTIONS

The study and the pharmaceutical evaluation of proteins (among them the AMPs), in pre-commercial character, requires the obtaining of these molecules in great quantities. For the assessment of aspects such as the different mechanisms of action and the structure/function relation, masses of peptides ranging from a few micrograms to many milligrams are required. In this context, the large-scale production of AMPs is the greatest challenge in prospecting and characterization studies of these peptides (Parachin et al. 2012).

The method of obtaining AMPs considered more advantageous in terms of cost and yield is one that explores recombinant or biofactory systems for the synthesis of heterologous proteins. In the last decades, several systems of heterologous expression have been developed with peculiarities that allow high levels of synthesis, modification and secretion of heterologous proteins (Parachin et al. 2012, Parachin and Franco 2014). This approach also allows the structural modification of peptides to carry out detailed studies on the function of these molecules.

More than 95% of the heterologous peptides are synthesized in heterologous expression systems using bacterial or yeast cells (Parachin et al. 2012). In these systems, the bacterial species most commonly used is *Escherichia coli*, a popular host because of its rapid growth, compatibility with a wide variety of expression vectors, low cost and high control of gene expression due to the wide knowledge about its genetic mechanisms and its physiology (Parachin and Franco 2014).

The isolation and prospection of several AMPs have been largely carried out by the group of researchers from the Center for Proteomic and Biochemical Analysis of the Catholic University of Brasilia (CAPB UCB). In addition to the structural and functional characterization of AMPs, the CAPB group specialized in its heterologous expression in bacterial system and in yeast cells. CAPB researchers have been successful in isolating the peptides from their natural sources or by their in silico redesign, which allows the addition, removal or replacement of amino acids with the aim of increasing antimicrobial activity or adding characteristics such as immunomodulatory capacity.

PROMISING AMPS WITH POTENTIAL THERAPEUTIC PROPERTIES AGAINST MULTIDRUG RESISTANT PATHOGENS

The antimicrobial peptide Cn-AMP1 was first isolated from coconut water (*Cocos nucifera*) in 2009, by Mandal and colleagues (Mandal et al. 2009). This small peptide is part of a peptide family with two other AMPs, called Cn-AMPs 2 and 3. All present nine amino acid residues with molecular masses between 0.8 and 1.3 kDa. When compared to the other two, Cn-AMP1 is the most active in vitro against the bacteria *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (MIC: 9 μM) and the fungi *Candida albicans*, *Trichophyton rubrum,* an anthropophilic dermatophyte fungus very common in skin diseases such as athlete's foot, and *Trichophyton mentagrophytes* (MIC: 18 μM) (Mandal et al. 2009, Santana et al. 2015). Other in vitro biological activity assays have shown that Cn-AMP1 exerts several alternative functions simultaneously to microbial control. The peptide was able to reduce the viability of different types of tumor cells, besides promoting immunomodulatory activity through the stimulation of cytokine secretion by laboratory-grown monocytes, without causing cytotoxicity to human erythrocytes and murine macrophages (Silva et al. 2012).

Another potent interesting antimicrobial peptide is Cm-p5, isolated from the mollusk *Centrichis muricatus*. Its prospection was performed after the tryptic fragmentation of a chromatographic fraction containing isolated peptides of *C. muricatus* analyzed by MS/MS, which generated a new candidate sequence (López-Abarrategui et al. 2015). From bioinformatics analyzes, a series of variant peptides was theoretically proposed based on the sequence, and these were previously evaluated against *C. albicans* fungus. Of these, Cm-p5 has demonstrated remarkable antifungal activity against strains of *C. albicans* with high medical importance. For purposes of illustration, the preliminary results of activity demonstrated MICs of 1 and 0.5 μg.mL-1, enough to obtain therapeutic effects. In addition, this peptide was also extremely active against *Cryptococcus neoformans* and *T. rubrum*. These values far exceed the activity of its precursor Cm-p1, reported in 2011. These results were the first to open the way for toxicity tests in animal cells and for the study of their mechanism of action (López-Abarrategui et al. 2015).

Clavanin A, a highly efficient AMP in the control of bacteria such as *P. aeruginosa*, was recently characterized by our group and demonstrated extremely high activity in the control of Gram positive and negative bacteria both in vitro and in vivo, from assays with mice (data in press). In addition, clavanin A was also efficient as a molecular stimulating agent for defense reactions of the attacked organisms (Silva et al. 2015). Therefore, the therapeutic potential of Clavanin A is promising, both in the direct antimicrobial action and in the immunomodulatory activity without aggravating the inflammatory response.

Among the AMPs isolated from plants, cyclotides are one of the most promising biomolecules with therapeutic potential against different classes of plant pathogens. These peptides were first described in the 1970s, isolated from different tissues such as leaves, stems, flowers, roots and barks of various plant families, such as Violaceae, Rubiaceae, Fabaceae, Asteraceae and Cucurbitaceae (Chen et al. 2005, Craik et al. 2006). The cyclotides are rich in cysteine and have between 28 and 37 amino acid residues, and have a unique characteristic: a cyclic structure, where the N-terminal region is linked to the C-terminal region in a circular chain, which distinguishes them (CisI-CisIV, CisII-CisV, CisIII-CisVI), and the presence of a single, highly conserved three-disulphide pattern.

This arrangement forms a motif known as Cyclic Cysteine Knot (CCK), which comprises a peptidic ring formed by two parallel disulphide bridges (CisI-

CisIV and CisII-CisV) and a transverse disulphide bridge (CisIII-CisVI), which stabilize the motif. In this way, from the combining of the cyclization and the CCK motif, the family of cyclotides groups vegetable cyclic peptides that share a three-dimensional structure that gives the molecules high stability and protection against proteolytic enzymes, as well as acidic conditions and chemical and physical degradation (Craik et al. 2006, Pelegrini et al. 2007, Pinto et al. 2016, Cunha et al. 2016).

Reports in the literature indicate the presence of large numbers of cyclotides in the Rubiaceae family and in a particular plant of this family, *Palicourea condensata* (Pinto et al. 2016). Cyclotides of a species of the central southern plateau of the same genus, *Palicourea rigida*, have been extensively studied by our group (Pinto et al. 2016). Curiously, this plant was little studied despite its widespread use in folk medicine in the region. Analysis of the protein content of *P. rigida* evidenced the presence of cyclotides in the inflorescence, peduncle and leaves, some of which appeared only in specific tissues, while others appeared in all explored parts. One of these peptides was sequenced and pioneered: Parigidin-br1, which contains 32 amino acid residues. This cyclotide was classified in the Bracelets subfamily due to the absence of a cis-proline in loop 5, and its function was evaluated *in vivo* against Lepidoptera (*Diatraea saccharalis*) neonates, and *in vitro* against insect SF-9 cells, presenting significant insecticidal and cytotoxic activities (Pinto et al. 2016). The scarcity of biochemical studies on *P. rigida*, added to its wide medicinal use, intensifies the interest for its evaluation as a source of Parigidina-br1, a molecule with clear potential of use for the protection of cultivated plants.

EXPRESSION OF RECOMBINANT DRUGS IN PLANTS - "MOLECULAR FARMING"

Since the development of recombinant DNA technology in the early 1970s, the recombinant production of proteins of pharmacological and industrial interest has been based on microbial (prokaryotic and eukaryotic) fermentation systems and culture of transgenic mammalian cells. Although these systems are highly efficient in terms of yield, they have some disadvantages in terms of authenticity (amino acid sequence identity and post-translational modifications), biosafety, and production costs, despite the recent development of strategies to minimize or cure them (Parachin et al. 2012, da Cunha et al. 2014).

One of the most promising alternatives for replacing the already established recombinant platforms is the use of plant systems as vehicles for the production of proteins used as diagnostic reagents, vaccines and drugs, also called plant-made pharmaceuticals (PMPs) or "drugs produced in Plants " (Tregoning et al. 2005, Cunha et al. 2017). This application represents a relatively recent strand of biotechnology called Plant Molecular Farming, the scope of which is the synthesis of heterologous polypeptides of pharmaceutical interest using the plant reactor machinery (Ma et al. 2005).

Plants can be attractive systems for the mass biosynthesis of drugs. Many plant species present desirable economic and qualitative aspects associated to the synthesis of "PMPs", such as low production costs and capacity to perform post-translational modifications required by complex AMPs. Plant reactors or "plant biofactories" are potentially one of the most economical systems for the large-scale production of this class of biomolecules. The average cost of production of "PMPs" represents about 10% of the total presented by systems based on yeast cells, being able to be up to 50 times smaller than that of bacterial systems. When compared to sophisticated and relatively expensive platforms such as mammalian cell culture, this difference may be even greater. It is estimated that the cost of producing a given recombinant drug equivalently expressed in tobacco seeds and mammalian cell suspension may

be up to 1000 times lower in the former than in the latter (Obembe et al. 2011).

The plants represent one of the types of biomass of lower cost of production in nature, requiring only soil, water and light for the production of large amounts of biomass, represented mainly by leaves and seeds. In contrast, cell culture of microorganisms and mammals requires the adoption of expensive fermenters and their manipulation under sterile conditions, something that considerably impairs the production process (Obembe et al. 2011, Davies 2010).

Since 1986, when the first relevant recombinant drug has been synthesized in transgenic tobacco plants - human growth hormone (hGH) - more than one hundred different heterologous proteins, including antibodies, antigens, hormones, molecular transporters, anticoagulant peptides and enzymes synthesized in different plant systems (Cunha et al. 2011a, b). The commonly used platforms to produce all these classes of molecules are the suspension of tobacco cells; cereal and legume seeds, such as maize and soya; oilseed, such as canola; potato tubers; the different species of leafy crops, such as lettuce and spinach, as well as fruits such as tomatoes and bananas (Egelkrout et al. 2012, Ma et al. 2003, Drake et al. 2017).

In addition to economic factors, the plants present very promising aspects regarding the quality of heterologous proteins and the biosafety of their use as reactors of PMPs. The main technical aspects of the production of therapeutic proteins in the different available systems are compared in Table I.

About thirty PMPs, including vaccines, antibodies and therapeutic proteins, have now reached the final stages of international pre-

TABLE I
Comparison between different production systems of heterologous proteins of pharmaceutical interest.

Type	System	Average cost	Timescale	Scalability	Quality (folding, subunit assembly, glycosylation)	Post translational modifications	Contamination risks	Storage cost
Microorganisms	Bacteria	Low	Short	High	Low	None	Potential presence of endotoxins	Moderate
	Yeast	Moderate	Moderate	High	Moderate	Similar	Low	Moderate
Cell culture	Mammal cell culture	High	Long	Very low	Very high	Identical	Potential presence of virus, prions and oncogenes	High
	Plant cell culture	Moderate	Moderate	Moderate	High	Similar	Low	Moderate
Transgenic multicellular organisms	Transgenic animals	High	Very long	Low	Very high	Identical	Potential presence of virus, prions and oncogenes	High
	Transgenic plants	Very low	Long	Very high	High	Similar	Low	Inexpressive
Transient platform	Plants infected with viral vectors	Very low	Short	Very high	High	Similar	Low	Inexpressive

Adapted from Ma et al. 2003.

marketing protocols and tests. Eight recombinant plant-derived products - seven laboratory reagents and one drug - have already been released. In 2012, the recombinant enzyme glucocerebrosidase - or taliglucerase alfa, secreted by suspension carrot cells and used for the treatment of Gaucher's neurodegenerative disease, became the first plant-biotech synthesized product to be released for commercialization in the United States (Tregoning et al. 2005, Shaaltiel et al. 2015, Drake et al. 2017).

Basically, there are two ways of expressing genes to produce PMPs: i) the stable transformation of the nuclear or plastidial genomes and ii) the transient expression of genes delivered by Agrobacterium or infectious transgenic viruses (Obembe et al. 2011).

Stable transformation of the plant genome is the most commonly used strategy for the production of recombinant PMPs and is applicable to a wide variety of plant species: from cereals to legumes, including leafy crops, oleaginous and fruit plants. This method advocates the stable integration of transgenes into the genome of the host plant and allows the continuous synthesis of the recombinant protein as an inheritable phenotypic trait, either generically or localized in a specific plant organ (Xu et al. 2012).

Transient gene expression is mediated by a bacterial or viral infective intermediate and is generally used to verify the efficiency of the gene construct activity employed in genetic transformation experiments and to quickly validate the structure and function of the recombinant protein (Loh et al. 2017).

However, infiltration of plant leaves - notably the *Nicotiana benthamiana* species - by vacuum or syringes using recombinant *Agrobacterium tumefaciens* suspensions may result in the transient transformation of several leaf cells and the attainment of high levels of gene expression a few days after the realization of the experiment, providing the routinely scheduling of PMP production at industrial levels (Gleba et al. 2014). Table II shows the main comparative characteristics between the two strategies of genetic transformation of plants for the development of PMPs, as well as the platforms available in each expression scenario:

The selection of the plant species destined to the production of PMPs represents one of the most important criteria aiming at the success of the molecular strategy of gene expression. Genomic, biochemical, physiological and even morphological features inherent in each species have a crucial influence on a number of factors, such as the yield of recombinant proteins, the ability to promote post-translational modifications in complex proteins, the structural stability of the polypeptide and the final cost of production (Stoger at al. 2002, Fischer et al. 2004).

Table III illustrates the properties and peculiarities of the main plant species used as recombinant protein bioreactors for the pharmaceutical and industrial sectors.

STRATEGIES FOR MAXIMIZING RECOMBINANT PROTEIN YIELD IN PLANTS

One of the most important factors determining the viability of producing heterologous proteins in plants is to obtain satisfactory amounts of the polypeptides. The absolute yield of production depends on the maximization of efficiency of all stages of gene expression and protein stability. Thus, the various strategies aimed at increasing the final amount of recombinant protein in plant reactors focus on gene transcription, post-transcriptional processing, translation and post-translational protein stability (Streatfield 2007).

STRATEGIES AT THE TRANSCRIPTIONAL LEVEL

Choice of promoters and terminators

To obtain high levels of transgene expression the two most important elements are the promoter and the transcription terminator (Ma et al. 2003).

TABLE II
Available platforms for the biosynthesis of PMPs.

	Stable transformation	References
Type	**Whole plants**	
	Stable nuclear transformation	
Key Features	Stable incorporation of exogenous genes into the nuclear genome	
	Stable inheritance of transgenes in successive generations	
	Used to obtain the majority of transgenic plants until today	
	Utilized commercially since 2014 in Japan by the company Hokusan for the production of interberry-alpha, a recombinant canine interferon-alpha produced in transgenic strawberry for the treatment of periodontal disease in dogs	Obembe et al. 2011 Drake et al. 2017
Advantages	Transmission of new characters as traits inheritable to the progeny	
	High scalability	
Disadvantages	Possibility of undesirable crosses in some species	
	Long cycle of production of some plant species	
	Usually poor levels of transgene expression	
Type	**Whole plants**	
	Stable plastidial transformation	
Key Features	Stable and simultaneous transformation of numerous copies of the plastidial genome	
	Exclusively maternal inheritance in many species	
Advantages	Natural biocontainment	Meyers et al. 2010
	Minimizing gene flow by out-crossing	
	High levels of expression (up to 70% TSP)	
Disadvantages	Limited to few species: tomato, lettuce, soybeans and eggplant.	
	Routine transformation of tobacco only	
	Variable protein stability	
Type	**Plant cell-suspension cultures**	
Key Features	Undifferentiated aggregates of plant cells dispersed and propagated in liquid medium	
	System used for the production of the first PMP to achieve commercial production status by the FDA, in 2012: Elelyso, the replacement enzyme glucocerebrosidase from the Israeli company Protalix, in addition to the commercial chicken vaccine against Newcastle disease virus (NDV) from Dow Agroscience	
Advantages	Fast, relatively inexpensive and high level of containment	Franconi et al. 2010 Drake et al. 2017
	Usually high purity production and low downstream processing costs when the PMP is secreted into the culture medium	
	Homogeneity of production	
	Low N-glycans addition heterogeneity	
Disadvantages	Need for sterile production conditions	
	Decreased levels of protein biosynthesis in stationary phase, due to proteolytic activity	
	Restricted to just a few crops such as tobacco, Arabidopsis, rice and carrots	
Type	**Transient expression systems**	
	Agroinfiltration method	
Key Features	Infiltration of tobacco leaves by suspension of *Agrobacterium tumefaciens* cells	
	Transference of bacterial T-DNA to a high number of leaf cells	
Advantages	Fast	Regnard et al. 2010 Loh et al. 2017
	High expression. levels.	
	Possibility of producing clinical grade pharmaceuticals	
Disadvantages	Rapid decay of gene expression after peak expression.	
	Inability to transfer transgene to progeny	

TABLE II (continuation)

	Stable transformation	References
Type	**Virus infection method**	
Key Features	Non-integrative method Based on the use of plant viruses, such as Tobacco Mosaic Virus (TMV) and Potato Virus X (PVX), as infectious carriers of transgenes Used to infect tobacco Used by the Large Scale company to obtain vaccines against B-cell non-Hodgkin's lymphoma	McCormick et al. 2008 Marsian and Lomonossoff 2016
Advantages	Fast, scalable and capable of obtaining high levels of recombinant protein biosynthesis	
Disadvantages	Restricted to tobacco Need for immediate processing due to protein instability	
Type	**Magnifection™**	
Key Features	Platform that combines the characteristics of the methods Agroinfiltration and Virus infection Developed by Icon Genetics Use of deconstructed viral expression cassettes, lacking protein coat sequences and viral motility proteins sequences Systemic delivery of genes is mediated by Agrobacterium Improved infectivity Increased levels of gene expression and biosynthesis of recombinant proteins greater than 80% TSP Fast	Gleba et al. 2014
Advantages	Capable of producing both small molecules, such as vaccine antigens, and large and complex IgGs Capable of co-expressing two or several polypeptides simultaneously Capable of assembling heterooligomeric proteins Ease of manipulation	
Disadvantages	Restricted to tobacco Need for immediate processing due to protein instability	

TABLE III
Plant species used as bioreactors of PMPs.

Species	Advantages	Disadvantages
	Leafy crops	
Tobacco	High biomass production Well established transformation and processing technologies High scalability Non-food and non-feed crop	Low post-harvest protein stability Presence of alkaloids
Lettuce	High yield of biomass Edible Useful for human vaccination	Low post-harvest protein stability
Alfafa	High biomass production Useful for animal vaccination Clonal propagation Addition of homogeneous N-glycans	Low post-harvest protein stability Presence of oxalic acid
Clover	High biomass production Useful for animal vaccination Clonal propagation Addition of homogeneous N-glycans	Low post-harvest protein stability
	Legumes	
Soybeans	Abundant biomass, possibility of transgene expression in seed coat High protein concentration in seeds High ratio of seed biomass /production cost	Usual low levels of transgene expression

TABLE III (continuation)

Species	Advantages	Disadvantages
Pea	High protein concentration in seeds	Usual low levels of transgene expression
Pigeon pea	High protein concentration in seeds	Usual low levels of transgene expression
Cereals		
Wheat	High protein stability during storage	Low yields Difficulties in processing and handling
Barley	High protein stability during storage	Low yields Difficulties in processing and handling
Maize	High protein stability during storage High biomass production Ease of processing and handling	Usual low levels of transgene expression
Rice	High protein stability during storage High biomass production Ease of processing and handling	Usual low levels of transgene expression
Fruits		
Tomato	Edible crop Containment in greenhouse	Expensive cultivation Low stability after harvesting
Tubers		
Carrot	Edible High protein stability in storage tissues High scalability Ease of purification and processing in cell suspension system	
Potato	Edible High protein stability in storage tissues	Usual low levels of transgene expression Needs to be cooked before consumption
Oilcrops		
Canola	Oleosin protein fusion platform Sprouting system developed	Low yields
Camelina sativa	Oleosin protein fusion platform Sprouting system developed	Low yields
Moss		
Physcomitrella patens	Ease of culturing under containment Facilitated clone propagation Allows secretion in culture medium Exhibits homologous recombination	Low scaling
Green algae and aquatic plants		
Chlamydomonas reinhardtii	Ease of culturing under containment Facilitated clone propagation Allows secretion in culture medium	Low scaling
Lemna	Ease of culturing under containment Facilitated clone propagation Allows secretion in culture medium	Low scaling
Model Plants		
Arabidopsis thaliana	High availability of mutants High genetic accessibility Ease of transformation	Low biomass

Adapted from Fischer et al. 2004.

Strong eukaryotic promoters present specific sites that facilitate the recognition of transcription factors and the RNA polymerase II. They can also be upregulated by specific enhancers that boost heterologous expression, providing high levels of mRNA production (Streatfield 2007, Tschofen et al. 2016).

The strongest constitutive promoters most widely used for heterologous expression in dicotyledons are 19S and 35S CaMV, derived from the 19S and 35S transcripts of cauliflower mosaic virus (CaMV).

In monocots, the ubiquitin 1 (ubi-1) promoter from maize is also very popular and efficient for the expression of recombinant proteins in cereals (Twyman et al. 2003).

Also, inducible promoters that allow external regulation by chemical and physical stimuli emerge as interesting tools to maximize gene expression at the transcriptional level (Twyman et al. 2003).

In this context, a peroxidase-activated sweet potato (*Ipomoea batatas*) promoter was used to enhance 30 times the expression of the *gus* gene in transgenic tobacco plants subjected to the presence of hydrogen peroxide and ultraviolet light than the 35S CaMV promoter (Kim et al. 2003). Another interesting example was the rapid induction of biosynthesis of heterologous proteins in tobacco using the endogenous promoter that controls the gene of the enzyme hydroxy-3-methylglutaryl-Coa-reductase (HMGR2), activated by mechanical stress induced by harvesting practices, a system developed by the late american company Crop Tech Corp (Padidam 2003).

Terminators widely used include the 35S CaMV, that of the genes *nos* and *ssu*, respectively of *A. tumefaciens* and pea (*Pisum sativum*) (Ma et al. 2003).

Minimization of transcriptional silencing

Several mechanisms lead to the interruption or inhibition of mRNA synthesis in plants, such as the presence of prokaryotic DNA sequences, typical of the plasmid backbone used in genetic transformation, DNA methylation, site-related "position effect" transgene integration into the plant genome, integration site structure, presence of multiple copies or "superfluous" copies of the transgene, sequences with potential for hairpin formation and double-stranded RNA, in addition to negative feedback from promoters - a common event when end products of gene expression are recombinant enzymes (Finnegan and McElroy 1994).

Some strategies have already proved effective in eliminating or reducing such problems, such as the use of "clean" vectors of prokaryotic sequences, absent from obstacles to the coupling of RNA polymerase II and that may lead to the formation of double-stranded mRNA; techniques for integrating single copies of the transgene into the plant genome; the addition of flanking sequences of the scaffold attachment regions and site-directed integration; the choice of germplasm with low methylation activity; in addition to the reduction of negative feedback from promoters by the expression of the target enzyme in a different cell compartment from which its substrate is found (Meyer and Saedler 1996).

STRATEGIES AT THE POST-TRANSCRIPTIONAL LEVELS

Minimization of post-transcriptional silencing

The processing of primary transcripts is crucial for obtaining high levels of heterologous protein. Although most of plant genetic transformation experiments utilize cDNA-like coding sequences originating from libraries constructed from properly processed mRNAs, the presence of introns in the

transgene can significantly increase the stability of mRNAs (Töpfer et al. 1993). This application has already been proven to increase the expression of endogenous genes in monocots, especially in maize plants (Töpfer et al. 1993).

Polyadenylation sites also exert great influence on the stability of mRNAs and on the levels of gene expression in plant cells.

Detection and elimination, where possible, of specific recognition sites that contribute to the decay of mRNAs in some terminators may also be used as a tool to avoid post-transcriptional silencing (Töpfer et al. 1993).

Translation optimization

The correct functioning of translation initiation is a limiting factor for the level of accumulation of heterologous proteins. The overlap of the translation initiation site with the Kozak consensus sequence of mRNA is a great optimizer of translation levels, even though there are small structural differences in these sequences in animals and plants, something important when the goal is to express human genes in transgenic plants (Kawaguchi and Bailey-Serres 2005).

The translation rate may also vary depending on the availability of specific amino acid transfer RNAs for certain codons in the plant cell. Optimization of codon usage through site-directed silent mutations or the production of previously modified synthetic coding sequences can be very useful in greatly enhancing the translation of eukaryotic mRNAs (Streatfield 2007).

The leader sequences of different plant transcripts have been shown to influence the increased levels of recombinant protein accumulation in plant bioreactors. They can be adapted on a case-by-case basis for different gene/host plant combinations to maximize translation efficiency (Streatfield 2007).

Post-translational protein stability

The levels of transcription and translation are related to the efficiency of biosynthesis of heterologous proteins. Another variable should be considered for the estimation of the production yield or accumulation of protein biopharmaceuticals: their level of degradation after biosynthesis, that is, their degree of stability (Stoger et al. 2005).

Molecular strategies that act on protein stability, coupled with the choice of promoters, correspond to the most efficient advances in terms of real increases in yield of recombinant biopharmaceuticals and include two distinct non-exclusive approaches: the use of tissue-specific promoters - mainly those associated with endogenous seed genes - and subcellular targeting of polypeptides ("protein targeting") (Abiri et al. 2015).

Tissue-specific promoters are regulatory sequences that restrict gene expression spatially to only one or more parts of the plant and may also indirectly regulate expression at a temporal level when the organ destined for the accumulation of heterologous proteins is associated only with a period of the culture cycle (eg flowers and seeds) (Capell and Christou 2004).

A number of tissue-specific promoters have been extensively characterized, such as those controlling the expression of a maize seed zein, wheat glutenin, rice glutelin and pea seed proteins (Ma et al. 2003) .

The expression of heterologous proteins specifically in seeds implies many advantages naturally provided by such organs. Unlike leaves, seeds are natural storage sites for high concentrations of reserve proteins used in embryo nutrition in the early stages of physiological development of the seedling (Stoger et al. 2005).

These organs present not only a suitable biochemical environment, devoid of phenolic compounds and low concentration of hydrolases,

but also presents specialized tissues for the highly stable protein accumulation for long periods of time even at room temperature, which reduces the need for special conditions of storage (Takaiwa et al. 2007).

As the costs of processing and purification are inversely proportional to the concentration of the product in relation to the plant biomass, the accumulation of high levels of heterologous proteins in a reduced volume leads to a significant decrease in the production costs of recombinant biopharmaceuticals (Stoger et al. 2002).

Protein Targeting

Subcellular targeting plays a key role in the levels of heterologous protein accumulation, since the cell compartment in which they are found directly influences the folding, assembling, and post-translational modifications processes, as well as preventing immediate degradation and interference of the polypeptides with cell metabolism - quite frequent events in the cytosol (Fahad et al. 2015).

N-or C-terminal signal peptides can be fused to heterologous proteins to target specific sites in the cell. These sequences can target proteins to the mitochondria, vacuoles, chloroplasts or retain them in the endoplasmic reticulum, and are generally cleaved after the arrival of the polypeptide of interest to the target organelle (Xu et al. 2012).

Commonly four subcellular targets are the main compartmentalization targets to produce biopharmaceuticals: the apoplast, the endoplasmic reticulum, the chloroplasts and the seed protein bodies (Daniell et al. 2001).

Depending on the molecular mass the polypeptide can be secreted or retained in the apoplast, which leads to important applications for suspension cell culture systems. Schillberg et al. (1999) compared the stability of identical whole antibodies whose accumulation was directed to the cytosol and to the apoplast of transgenic tobacco leaves and found that the secretory pathway constitutes a set of environments more suitable for the folding and assembly of this type of complex protein, since levels of accumulation in the apoplast were much higher than those of the cytosol.

The main disadvantage of addressing to the apoplast is the fact that heterologous proteins are required to be processed before in the Golgi complex, where the addition of typical glycans occurs, which can lead to loss of structural and functional authenticity of the polypeptides (Daniell et al. 2001)

The passage of proteins through the reticulum is the initial destination of polypeptides forwarded to the secretory pathway and the retention of these molecules in the lumen of this organelle has been shown to be more advisable for the accumulation of larger amounts of recombinant proteins in plants than the apoplast itself and the cytosol (Zimmermann et al. 1998).

Conrad and Fiedler (1998) determined that the amount of recombinant antibodies fused in the C-terminal portion to the tetrapeptide K/H DEL - one of the most popular peptide-signals currently employed for the retention of heterologous proteins in the lumen of the endoplasmic reticulum - was 2 a 10 times greater than those expressed in different plant species in the absence of the signal peptide.

The use of K/H DEL also accounted for a marked increase in the accumulation of DIP B protein in leaves cells of Arabidopsis thaliana, resulting in 8.5% of total soluble proteins in these organs (Yang et al. 2005).

Polypeptides expressed in cereal seeds remained stable up to three years after storage at room temperature, with no significant loss of activity due to the low proteolytic activity in these compartments (Larrick and Thomas 2001).

Another advantage of so-called protein targeting for the endoplasmic reticulum of seeds is the fact that proteins retained in this organelle do not undergo processing in the Golgi complex,

a fundamental aspect for the production of heterologous proteins and recombinant antibodies in particular (Stoger et al. 2002).

Protein targeting for chloroplasts is also an interesting strategy to increase levels of recombinant protein accumulation. In the chloroplast lumen there are chaperones capable of assisting in the folding and assembly of heterodimeric and oligomeric proteins, in addition to low concentrations of hydrolases and peptidases, which makes this environment relatively stable for the accumulation of proteins of interest (Daniell et al. 2001).

However, since the machinery of gene expression in chloroplasts resembles that of prokaryotes, most post-translational modifications, such as glycosylation, cannot be performed on this organelle, except for the formation of disulfide bonds (Tschofen et al. 2016).

Protein bodies, in turn, are extensions derived from the endoplasmic reticulum specialized in the accumulation of reserve proteins in seeds of several species of grain-producing plants. They function as cisterns that occupy a large cytoplasmic volume and evolutionarily have undergone adaptations resulting in the high capacity to compartmentalize large volumes of proteins and to maximize the integrity of these polypeptides (Zheng et al. 1992).

These globular inclusions are found in cotyledonary cells of tobacco seeds, legumes such as soybeans and beans, as well as grasses such as wheat and barley, and originate now when biosynthesis of reserve proteins begins in the tissues responsible for nourishing the embryo (Yoo and Chrispeels 1980). In this way there is a significant increase in the number and availability of protein bodies throughout the process of physiological maturation of the grains of these species.

Since these highly specialized vacuoles do not undergo any type of fusion with lysosomes, their lumen presents near-neutral pH and practically the absence of aminopeptidases, factors that characterize them as a subcellular environment where protein degradation is minimal and an excellent target for the addressing heterologous polypeptides when the main goal of genetic transformation is to maintain the stability of these molecules (Takaiwa et al. 2007).

MAGNIFECTION™: A METHOD FOR THE MASS EXPRESSION OF HETEROLOGOUS PROTEINS AND PEPTIDES IN *NICOTIANA BENTHAMIANA*

As responsive elements of innate defense systems of microbes, plants and animals, AMPs are naturally synthesized at low levels (Watkins and Bonomo 2016, Uhlig et al. 2014). To maximize peptide biosynthesis, genetic engineered bacteria and yeast cells are frequently explored as vehicles for the recombinant production of bioactive AMPs (Perry et al. 2016, Nagel et al. 2016). To this day, many different AMPs have been synthesized in *E. coli* and *Pichia pastoris* (Perry et al. 2016). Apart from the high therapeutic potential presented by recombinant AMPs, limited investment of companies and drawbacks in terms of poor yield, low quality and unsatisfactory *in vivo* activity restricted the commercial development to few promising AMPs. Besides production limitations, some of them reached advanced clinical trials prior to commercialization.

Among the most important factors that limit the recombinant production of AMPs in microbial systems are the inner toxicity of the peptide for the host cells – for many AMPs, even low concentrations can be fatal for the microbe – and low quality of product in terms of post-translational modifications. Under such circumstances, plant appear as interesting alternative systems for the production of recombinant AMPs (Kosikowska and Lesner 2016, Nagel et al. 2016).

Although plants perform a vast arsenal of post-translational modifications, low levels of recombinant biosynthesis of peptides are common, resulting in low quantities of purified products. In addition to such limitations, plant systems for

expression of heterologous proteins have undergone enormous technical improvement in recent years. The discovery of strong promoters associated with increased gene expression, strategies and elements that stabilize primary transcripts, and optimization of codons and methods that maximize posttranslational stability have contributed to dramatic increases in the final amounts of proteins accumulated in transgenic plants and In suspension of plant cells (Obembe et al. 2011). Despite significant improvements, limitations such as the usually low levels of expression and the long time to obtain enough protein quantities for the first biological assays still restrict recombinant production in these systems (Xu et al. 2012).

The most interesting and recent alternative to circumvent both limitations is the use of viral vectors of gene carrier plants of interest that provide high levels of transient expression within a few days after inoculation. The Magnifection™ system integrates methods for expressing transgenes of interest using viral vectors specially constructed to provide high transcription rates, high speed in obtaining the first milligrams of heterologous protein and high scalability (Gleba et al. 2005).

In essence, the the Magnifection™ strategy advocates total infiltration of adult plants of *N. benthamiana* with a diluted suspension of *Agrobacterium tumefaciens* containing RNA replicon encoding T-DNAs, usually based on tobacco mosaic virus (TMV). In this process, the bacterium assumes the initial viral functions of primary infection and systemic movement, while the viral vector promotes short scattering (cell to cell), signal amplification, and expression of the transgene of interest at high levels. Thus, few adult tobacco plants allow the preliminary optimization of dilutions of the suspensions and the obtaining of milligrams or grams of the heterologous protein (Gleba et al. 2014) (Figure 1).

The Magnifection™ technology combines the advantages of standard expression systems (viral, bacterial and plant) and presents three major advantages when compared to other platforms. The first one is the use of efficient viral vectors with a design that allows high gene expression. This allows high rates of transgene expression, especially with respect to the increase of gene transcription, guaranteed by the presence of strong viral promoters. The presence of introns in the expression cassette also allow for the correct migration of the mRNA into the cytosol of the plant cell for the massive production of recombinant proteins (Gleba et al. 2004, 2014).

Secondly, Agrobacterium promotes the genetic transformation of somatic cells of *N. benthamiana* leaves in a systemic way. With cell-to-cell bacterial turnover, there is no need to increase the viral expression cassette with superfluous genes, such as viral motility coding genes, for example. This increases the spread of transgenes integrated into the plant chromosome and does not allow metabolic shifts to the biosynthesis of additional proteins without utility, concentrating the entire production effort on the transcription and translation machinery (Gleba et al. 2004, 2007).

Finally, the systems allow unprecedented speed and efficiency of accumulation of transcripts equal to those of a plant retrovirus and consistently high production yields at low cost (Gleba et al. 2005). The high scalability of agroinfection of plants with transgenic *A. tumefaciens* is due to the high inoculation yield – 1 litre of bacterial culture medium is sufficient for the infection of more than 10 kg of leaves - and to the rapid peak of gene expression, which normally occurs between the seventh and the eighth days after the agroinfiltration (Figure 2).

In a synthetic way, the Magnifection™ system allows the industrial production of proteins and peptides without the genetic transformation of germ cells, which leads to rapid and safe protein synthesis.

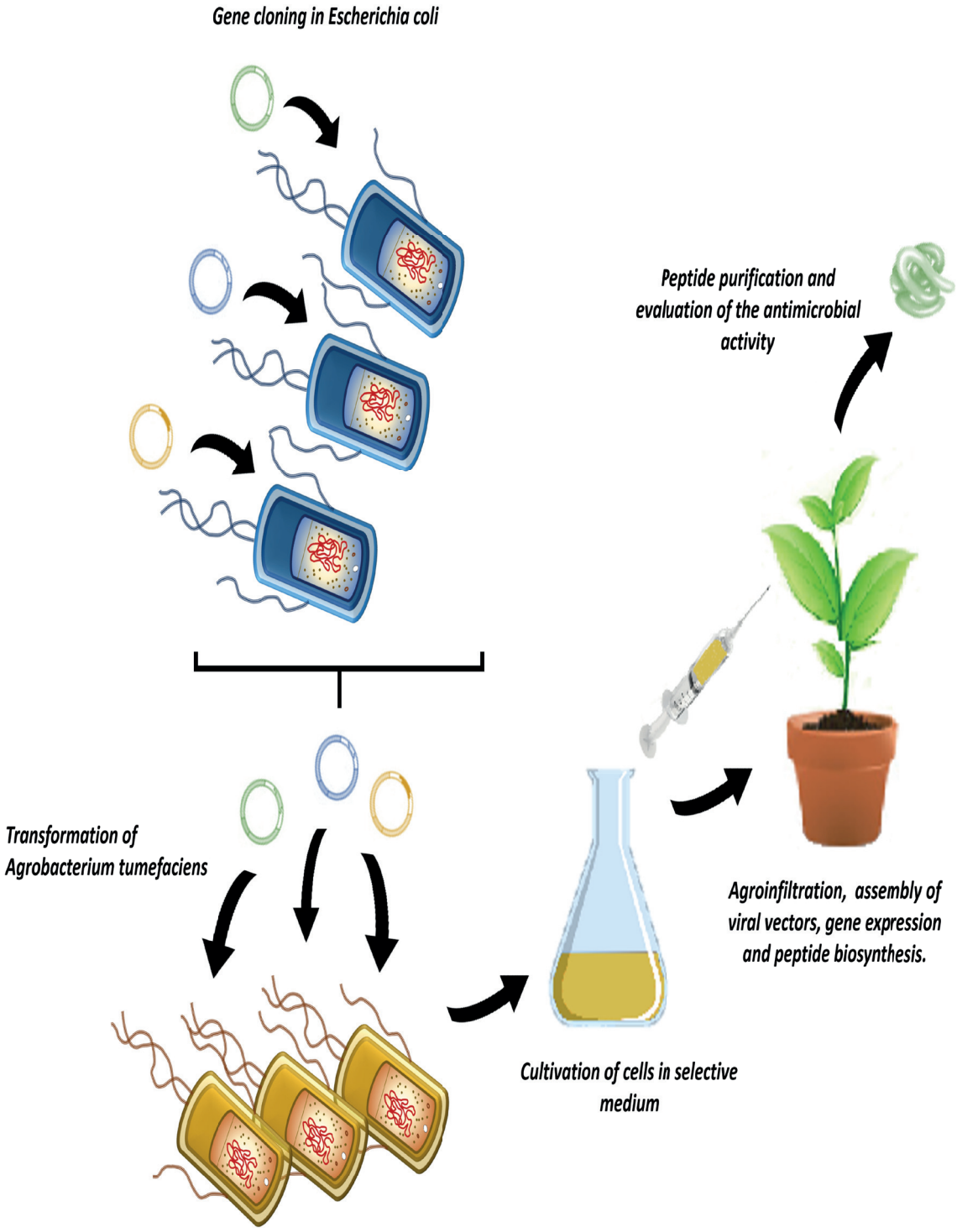

Figure 1 – Agroinfiltration method for the large-scale biosynthesis of recombinant AMPs. After gene cloning using *E. coli* cells, pro-vectors carrying the 3`extremity of the expression cassette (green vector), the 5`extremity of the expression cassette (blue vector) and the gene that codifies the integrase for the extremities recombination (yellow vector) are inserted in *A. tumefaciens* cells, followed by cultivation in LB medium. The bacterial culture is then injected in the downside of *N. benthamiana* leaves. After 5 to 8 days, yields of recombinant AMPs can be obtained from harvested leaves after extraction and purification.

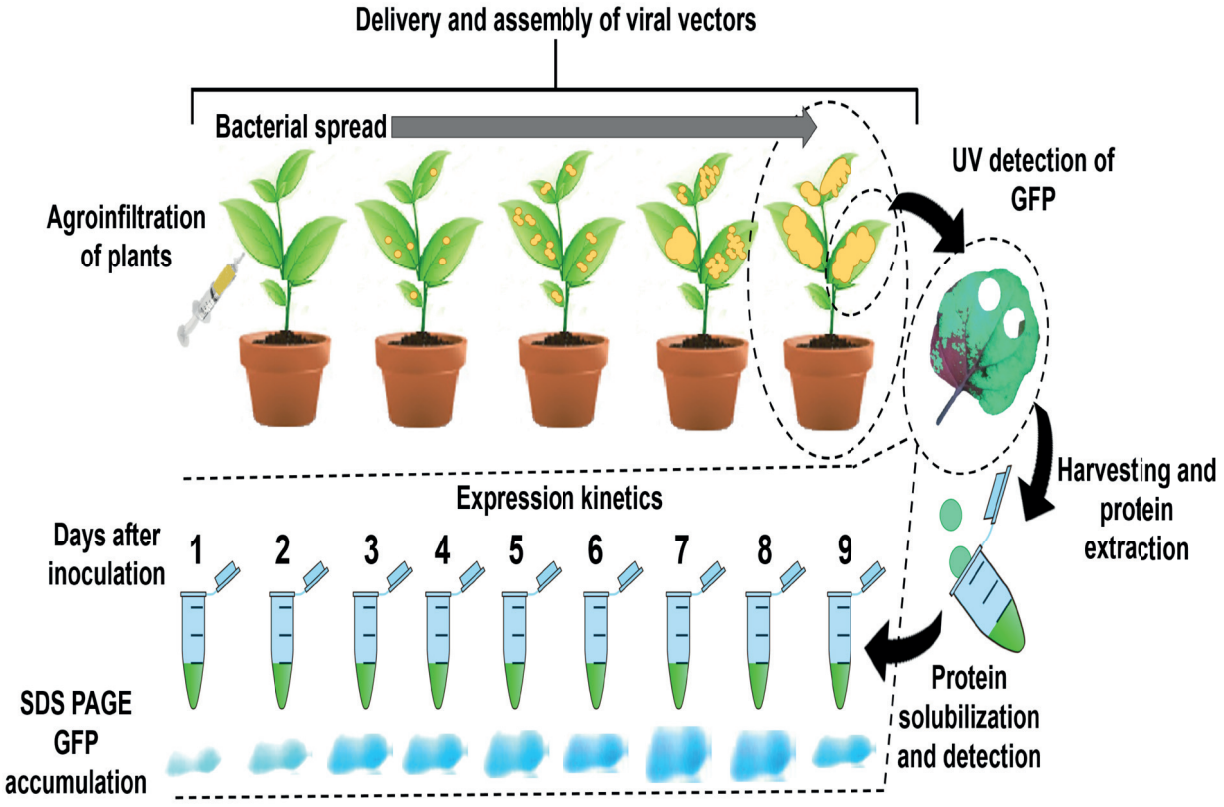

Figure 2 - Expression kinetics of foreign genes in *N. benthamiana* leaves. The gene delivery strategy mediated by *A. tumefaciens* allows the efficient spread of AMPs coding genes and integration to the host cells. Potent viral promoters induce high expression rates of the Green Fluorescent Protein (GFP) from *Aequorea victoria* gene, a frequent reporter gene used for experimental validation. The peak of expression is about the seventh or eighth day after inoculation.

As an alternative to the stable transformation of nuclear and plastid genomes, transient expression consists of the transient transformation of somatic cells by genes systemically carried by Agrobacterium and in the amplification of protein accumulation based on viral vectors in leaf cells. The Magnifection™ system uses the strategy of "deconstructed" viral vectors, i.e., vectors containing lean expression cassettes that dispense superfluous elements related to protein translation, the assembly of mature virions and their spread over long distances, the reprogramming of metabolism Host plant, inhibition of silencing and etc. Many of these actions are delegated to *Agrobacterium*, notably spreading, the host plant (Marillonnet et al. 2004, 2005).

The industrial scale can be easily obtained by means of a simple apparatus for vacuum agroinfiltration of whole plants, wherein the incubation time for viral amplification and peak expression does not exceed 10 days. Frequently, 5 grams of heterologous proteins are obtained per kilogram of infiltrated dry matter, and levels of expression equal to 80% of total soluble proteins are common in greenhouse (Gleba et al. 2004).

Since 2010, the Magnifection™ system has been used by the Canadian company Medicago for the industrial production of a vaccine against influenza caused by the H1N1 virus in the United States. A $ 21 million funding agreement was signed between Medicago and the American Defense Advanced Agency Agency (DARPA) to produce 10

million doses / month in the event of a pandemic. The vaccine is undergoing phase II clinical trials (in a total of IV) and tends to join another vaccine against the viral variant H5N1, produced with the same system (Holtz et al. 2015).

Today, many antigens have already been synthesized using the Magnifection™ system, as shown in Table IV.

A series of modern viral vectors based on TMV, called PICHs, are optimized for the expression of pharmacological proteins. Such plasmids, referred to as pro-vectors, contain tobacco introns systematically arranged throughout the molecule for processing in the nucleus and recombination sites that allow manipulation of the DNA still within the cell prior to conversion into a hybrid amplicon of RNA. DNA molecules containing the genes of interest can be efficiently recombined in plant, using site-specific recombinases such as Cre or the Integrase C31 from *Streptomyces sp*. (Gleba et al. 2014). To obtain an RNA without the recombination sites, engineered as introns by the RNA processing machinery of the nucleus. In summary, the process advocates the in vivo assembly of DNA pro-vectors by simple mixing and co-infiltration of plants with Agrobacterium cells that carry the components of the vector separately (Figure 3).

In this way, a series of three pro-vectors must be used simultaneously in the experiments to obtain high levels of recombinant expression. This approach allows rapid assembly and expression of protein variants by the combination of peptide signals, facilitating purification tails, binding domains and transit peptides, in a versatile arrangement that provides milligrams of proteins in only a few days (Gleba et al. 2004, 2014, Marillonnet et al. 2004, 2005). Due to all the properties exposed, the Magnifection™ system can be an excellent platform for the large-scale biosynthesis of the antimicrobial peptides Cn-AMP1, Clavanin A, Cm AMP-5 and Parigidin-br1.

TABLE IV
Examples of transiently expressed antigens using the Magnifection™ system.

Antigen	Disease/target	Status	Ref
Der p 1	Allergy	In vitro	Lienard et al. 2007
Protective antigen Der p 2	Antherax	Animal pre-clinical trial	Koya et al. 2005
L1 major capsid protein	Cervical cancer	Animal pre-clinical trial	Lenzi et al. 2008
VCA antigen	Epstein–Barr virus	In vitro	Lee et al. 2006
HSP-A	*Helicobacter pylori*	Phase I/II clinical trial	Gu et al. 2005
VP1	Foot and mouth disease	Animal pre-clinical trial	Wu et al. 2003
Hepatitis B/C	HBsAg (Hep B)	Animal pre-clinical trial	Thanavala et al. 1995
F1-V	Plague	Animal pre-clinical trial	Del Prete et al. 2009
SARS-CoV-S1	SARS	Animal pre-clinical trial	Pogrebnyak at al. 2005
Tet-C	Tetanus	Animal pre-clinical trial	Tregoning et al. 2005
Type 1 diabetes	GAD65	Animal pre-clinical trial	Ma et al. 2004
HIV p24 capsid protein HIV	AIDS	In vitro	Zhang et al. 2002

Adapted from Cunha et al. 2017.

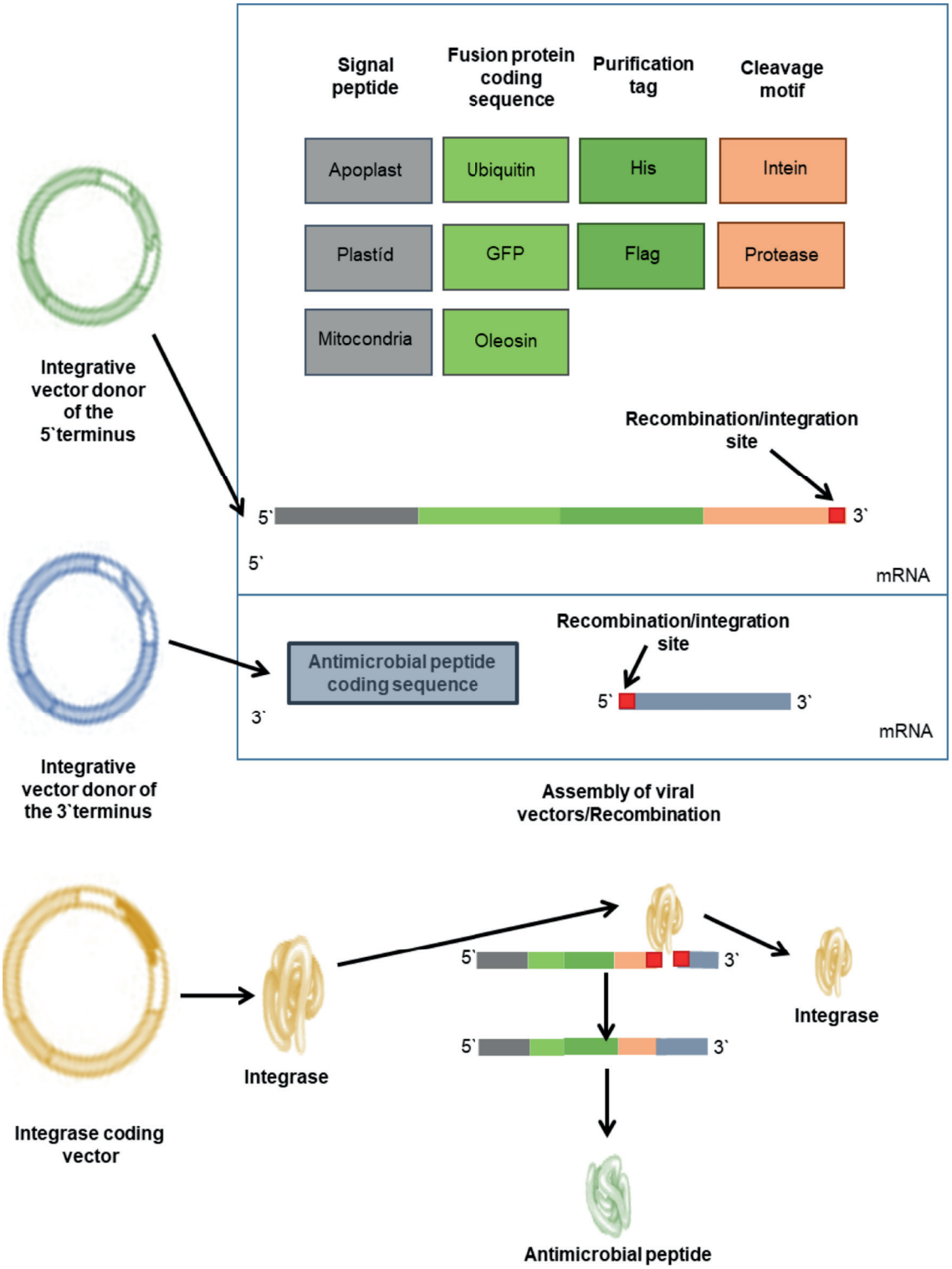

Figure 3 - Pro-vector system for assembly of gene expression modules. Two viral vectors, donors respectively of the 5 'and 3' ends, are specifically recognized by the enzyme Integrase C31 from Streptomyces sp. The enzyme catalyzes the recombination between the fragments by the pairing of homologous bases at the recombination sites. The final hybrid fragment presents all genetic elements for large-scale gene expression and for the addressing / purification of recombinant AMP.

CONCLUSIONS

Recombinant expression of the AMPs using the Magnifection™ system will provide subsidies for the systematization of large-scale expression of molecules with very high therapeutic potential and ability to inhibit a wide range of pathogens that threaten human health. Pro-vector combinations represent a great advance concerning the production of recombinant therapeutic molecules. As a consequence of their self-assembly ability, viral vectors may be used to study the expression of several other AMPs extracted from other plants, for example, thionines, snakines, heveins, defensins, lipid-transferring proteins and other cytotoxic agents. Such improvements allow the production of large quantities of AMPs sufficient to test new antimicrobial control functions with potential application in agriculture, pharmacology and food processing.

The massive biosynthesis of AMPs transiently accumulated in *N. benthamiana* leaves has great scientific impact, since it creates conditions for the systematic study of the rapid and mass expression of antimicrobial peptides in a cheap and safe system.

In addition, the industrial scale production of AMPs in a simple recombinant system with high capacity of production scheduling, besides the cultivation of greenhouse plants under conventional growth conditions, with only water, light and fertile soil, represents a great economic potential for the generation of efficient and cheap products with high value.

It is worth mentioning that this transient expression approach using deconstructed viral pro-vectors is a pioneer technology that represents the cutting edge of what is most sophisticated in terms of the use of heterologous vegetative systems for therapeutic purposes.

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